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Testing the Environment for Dispersed Mutagens: Use of Plant Bioconcentrators Coupled with Microbial Mutagen Assays

by William S. Barnes* and Edward J. Klekowski, Jr.*

Mutagens dispersed in ecosystems are usually in low concentration and episodic in occurrence. The possibility of detecting such dispersed mutagens by utilizing indigenous bioconcentrator organisms coupled with a microbial mutagen assay may offer a useful screening protocol. There are numerous examples of plant and animal species which concentrate toxic substances from the environment. Body extracts of these bioconcentrators can be suitably fractioned and tested for mutagens with various microbial mutagen assays. The fractions may be tested with a broad range of microbial assays covering numerous genetic end points as well as both with and without mammalian microsomal activation. This kind of environmental screening has an advantage over physicochemical techniques, in that sampling techniques are simpler and a wider chemical spectrum can be screened. There are problems inherent with testing a complex biological extract, however. If a reversion assay is used, the metabolite necessary for growth may be present. Toxins may be introduced, either concentrated from the environment in the same way as the mutagen, or produced by the concentrator itself. Finally, the concentrator may also produce an endogenous mutagen which will give spuriously active extracts. Methods for minimizing some of these difficulties are discussed.

Introduction

A great deal of epidemiological evidence suggests that cancer is an environmental disease (*1*). Current research suggests that carcinogens/mutagens may be common in certain environments (*2–10*) and that there is a great need to develop assay protocols to detect dispersed mutagens in ecosystems. Three papers in this workshop have dealt with such assays. Van't Hof and Schairer (*11*) have discussed the *Tradescantia* system (in this case a very sensitive mutagen assay organism is exposed to a component of the environment for a period of time) and Klekowski has described an assay based upon the detection of post-zygotic mutational damage in indigenous fern populations (*12*). For the advantages and disadvantages of these assay protocols, the reader is referred to these papers. In this communication we shall discuss an assay that utilizes certain indigenous or introduced components of an ecosystem to concentrate dispersed mutagens from the environment. Extracts are made from these bioconcentrators and tested for mutagenic activity with microbial mutagen assays. This is termed a coupled assay, since it couples an accumulator of environmental mutagens with a microbial system for detection. This approach has already been used successfully by Parry et al. (*13*) to detect mutagens in a marine environment.

Advantages of Coupled Assays

Two primary advantages accrue from bioconcentration of mutagens in contrast to physicochemical (abiotic) methods. Abiotic methods generally are based upon removing specific compounds and thus suffer either from excessive specificity, in that only a narrow band in the chemical spectrum is removed, or, if gauged more broadly, from problems in acquiring a sufficient amount of concentrate. A useful bioconcentrator would lessen both these problems. Secondly, mutational incidents may be episodic in time and space so that an intricate sampling procedure would be required for detection with an acceptable degree of certainty. The use of a bioconcentrator, however, provides a single, integrative sample indicative of long-term trends at the site of collection.

* Department of Botany, University of Massachusetts, Amherst, Massachusetts 01003.

The Bioconcentrator

The use of biological concentrators in ecosystem monitoring is not new. Mussels have been shown to accumulate heavy metals (*14, 15*), hydrocarbons and benzo(a)pyrene (*16–18*). Haug, Melsom, and Omang (*19*) showed that *Ascophyllum nodosum* collected on the coast of Norway sometimes contained increased levels of zinc, copper, lead, and cadmium and that this increase seemed related to industrial proximity. Concentrations varied from approximately 50 to 100 times greater than found in plants from unpolluted areas. Seeliger and Edwards (*20*) found that four species of littoral algae collected from Raritan Bay, New York contained copper and lead levels from 10^3 to 10^4 times higher than the surrounding sea water. Despite these concentration factors the mechanism of accumulation is not known; different mechanisms may operate for the concentration of different heavy metals (*19*). However, cation exchange has been established for some metals. Haug and Smidsrød found that the accumulation of cadmium, magnesium, and strontium ions was dependent on the amount and composition of alginates in the plants and on the relative concentrations of the ions competing for exchange sites (*21*).

If concentration does occur by surface exchange of cations, the implication for accumulation of mutagen/carcinogens is great. Miller and Miller (*22*) pointed out that there was a strong correlation between the mutagenicity, carcinogenicity and electrophilic reactivity of a chemical. It is generally accepted now that carcinogens are electrophilic and act by chemical bonding to nucleophilic portions of DNA (*23*). It may be, then, that the same properties which lead to carcinogenicity also will result in efficient adsorption of such molecules to nucleophilic sites on algal cell walls. One possible pitfall could be the fact that many carcinogens only become electrophilic after metabolic activation, hence may not be strongly adsorbed as procarcinogens.

Aquatic angiosperms also may be useful bioconcentrators. Water hyacinth (*Eichhornia crassipes*) has been used to remove phenols and a wide variety of heavy metals from water (*24–27*). Bingham and Shaver (*28*) found that the herbicide diphenamid was quickly removed from water by parrotfeather (*Myriophyllum brasiliense*) and water hyacinth. Such plants are beginning to be studied only now, and interest, at this time, seems to be centered on water purification. Nevertheless, it seems possible to use any good concentrator in a dual role for as it removes chemicals from the water it also becomes a monitor of the mutagenic activity present.

Assay for Genetic Activity

The second part of any coupled system is the organism used to screen for genetic activity. The most widely used systems are strains of *Escherichia coli* or *Salmonella typhimurium* containing frameshift or base substitution mutations which revert in the presence of mutagens specific for these effects (*29, 30*). A strain of *S. typhimurium* is available which detects the induction of deletions (*31*). Forward mutation systems also are available in *E. coli, Saccharomyces cerevisiae,* and *S. pombe* (*13, 32–34*). Other genetic end points such as mitotic crossing over, gene conversion and non-disjunction can be assayed with *S. cerevisiae (32, 35*). It might be possible to use *Drosophila* in tests for the induction of sex-linked recessive lethals, deletions and nondisjunction. By coupling two systems, the range of genetic end points which can be tested is greater than for any one system alone.

False Positives and False Negatives with the Coupled Assay

If a bioconcentrator is to be used, there are problems that may be encountered beyond the standard ones of finding the organism at a suitable spot and of assuring that it is a sessile long-time "resident" of the locale. The extract obtained must be free of endogenous mutagens and either endogenous or exogenous microbial toxins. If the assay being used depends on correction of an auxotrophic phenotype, the extract must not contain any supplement capable of overcoming this deficiency. Finally the metabolism of the concentrator must not degrade or activate substances so as to create genetical artifacts. A problem which may be encountered in extracting mutagens is the possibility of concurrently removing substances which interfere with an assay by allowing auxotrophic cells to grow and divide when only revertants to prototrophy should be capable of doing so. If this happens, a larger population of cells occurs on the plate, and if the mutation rate does not change, there will be an apparent, but spurious, increase of revertants over controls (the generation of false positives). This problem has not been studied, probably because in tests of pure chemicals, the generation of false positives will rarely occur in this manner. However, when testing extracts, it is a necessity to know exactly how reversion frequency is effected.

As a first step toward doing this we have determined the effect of feeding on reversion frequency

in the Ames test. The Ames test measures reversion frequency from histidine auxotrophy to histidine independence in a series of *Salmonella* strains. These strains differ both in the type of lesion they carry in the histidine locus and in the mutations incorporated into them to increase permeability and sensitivity. The two most sensitive strains are TA98, a frameshift mutant, and TA100, a base substitution mutant. Since both are histidine auxotrophs, it is possible to increase the level of feeding by appropriate supplementation with histidine. When this is done, it is found that TA100 gives a much more dramatic increase in prototroph frequency than TA98. If variance about the mean is similar for the two strains, TA100 could be considered more sensitive to false positives generated by feeding. Therefore, it is very important to detect such an occurrence. The usual method of doing this, by visually scanning the background lawn, is unreliable. One reason is that the thickness of the agar in a plate often varies, giving different impressions of the amount of growth. Furthermore, when crude extracts are used there is usually added turbidity, further confusing the issue. It will be seen that a quantitative measure of auxotrophic growth is a necessity. It might be assumed that extract controls from unaffected areas might be used to counter the effects of feeding. However, the influence of physiological state and external environment on amino acid content of the extract is unknown. Besides this, there is no way to be certain that a site close enough to the test area to be a reasonable control is not itself contaminated.

It is possible to measure the growth of the auxotrophic background directly. This is done by cutting pieces from a Pasteur pipet and using these to remove cores of agar from the plate. A sterile cotton swab is inserted into the tube and used to dislodge the cells and top agar. The core is pushed out into a culture tube containing sterile saline, vortexed, diluted, and plated on complete medium for cell counts. These techniques give reproducible results with very little error when cell count is regressed on histidine concentration. The percentage of the true number of cells in the background lawn which were retrieved is not really important so long as it is consistent and correlates with the amount of extra histidine provided. However, we can calculate that there are at least 1-2 $\times$ 10^9 cells per plate, and this suggests that our numbers approximate the true population size. Once cell counts are obtained, it is possible to plot these against reversion frequency (Fig. 1). This gives us a basis to determine the revertant frequency expected on *any* plate given a certain background population size. If there is additional supplementation, then it should be possible to cancel the increases due to feeding.

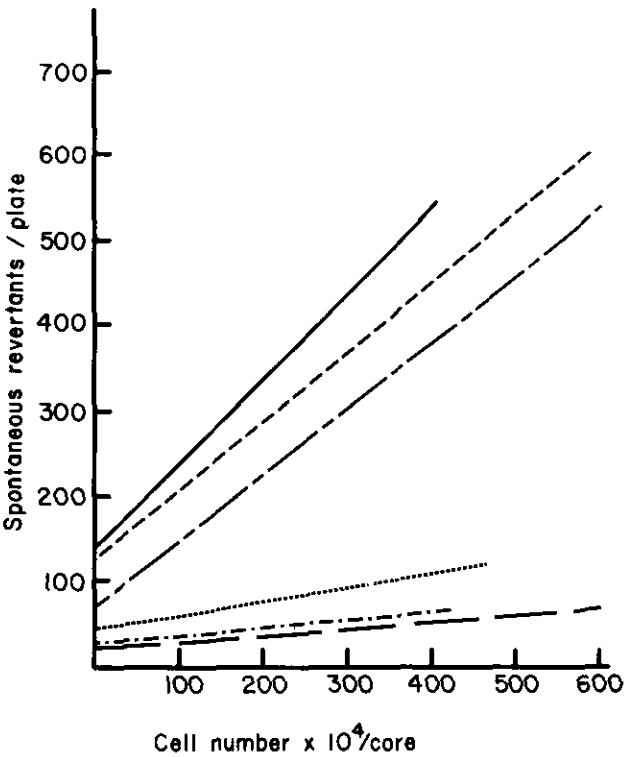

FIGURE 1. Spontaneous revertants plotted against cell number in the auxotrophic background lawn. Upper three curves, TA100; lower three curves, TA98.

Another way to minimize the effect of feeding is to extract in a solvent in which the interfering metabolite is insoluble. Thus, in the Ames test, which depends on reversion from histidine auxotrophy, an organic solvent is used because histidine is a polar molecule (*36*, *37*). Another method is to remove histidine using resin adsorption techniques (*38*). Unfortunately these methods also may remove mutagens; benzo(a)pyrene, for example, is removed nearly as efficiently as histidine.

Endogenous microbial toxins produced by the plant bioconcentrator itself may be a problem. A list has been made of plants which might be useful aquatic or terrestrial bioconcentrators but for reportedly bactericidal properties. Included are only those plants which are relatively common and are reported to act against gram negative bacteria such as *E. coli* and *Salmonella*, or yeast. The study of antimicrobial agents in higher plants seems to have become rather unfashionable since the early 1960's so the most recent, fairly comprehensive review is that of Nickell (*39*). According to Nickell, the following plants may contain microbial toxins: *Acer spp.* (especially *A. saccharinum, pseudoplatanus* and *rubrum*), *Achillea millefolium, Ambrosia artemisiifolia, Asclepias spp., Aster spp., Daucus*

carota, Eichhornia crassipes, Erigeron spp., Ginkgo biloba, Nelumbo lutea, Nymphaea odorata, and *Nuphar variegatum*. Yeast appears to be the least susceptible and many times is not inhibited when gram negative bacteria are. Curiously, *Ginkgo* inhibits yeast but not bacteria.

More recently Mitscher (*40*) listed the following species as bactericidal: *Acer saccharinum, Arctium minus, Berberis thunbergii, Chrysanthemum frutescens, Populus alba, Rhus copallina, Syringa vulgaris* and *Thalictrum spp.* Also, Su et al. (*41*) tested many water weeds, including *Nuphar variegatum, Nymphaea tuberosa,* and *Lemna minor,* and found that none of these extracts inhibited *E. coli.* Since Nickell reported that *Nuphar* and *Nymphaea* were bactericidal, more work is needed on these potentially important bioconcentrators.

In addition to crude plant extracts, some specific antimicrobial substances are known. One such group is the cyanogenic glycosides which liberate free cyanide upon hydrolysis. These toxins are found in *Prunus, Pyrus,* and *Cotoneaster* of the Rosaceae and *Andropogon* and *Panicum* in the Gramineae (*42*). Tannic acid, a common plant product, is reported to have strong antimicrobial action against *E. coli.* (*41*). The above list suggests that microbial toxins are ubiquitous in plants and thus many plants will not be useful as bioconcentrators. It should be noted, however, that Mitscher, in repeating some of the older experiments, found only 20% of the data reproducible. Thus, the true picture of toxicity may be somewhat brighter.

A related problem is one of mutagens endogenous to the concentrator being used. The subject of mutagenic and carcinogenic plant secondary compounds has been reviewed recently (*43–45*) and further information can be obtained from simultaneous use of a survey of carcinogenic compounds (*46*) and Gibb's *Chemotaxonomy of Flowering Plants* (*42*). Up to this time, there has been very little work done on the mutagenicity of plant secondary products. Consequently, much of the available information concerning the genetic activity of these substances comes from studies of their carcinogenic effects. Since carcinogenicity is believed to equate with mutagenicity in microbial tests, especially if microsomal activation is used, the two have been treated as synonymous. In discussing certain plants or taxa which might prove troublesome in this regard, a distinction has been maintained between these two types of information, however.

Bjeldanes and Chang (*47*) assayed the mutagenic activity of several closely related flavonoids and found quercetin and quercetin pentaacetate to be mutagenic. The flavonoids are a very large group of plant metabolites and it is conceivable that more of them may be genetically active when tested. The mutagenicity of quinoline and many methylated and chlorinated derivatives has been shown also (*48*). 4-Nitroquinoline-1-oxide has long been known as a mutagen and, not too surprisingly, 6-nitroquinoline and 8-nitroquinoline also are found to be active. Interestingly however, isoquinoline and 3-methylisoquinoline are not mutagenic, even though they differ only in the placement of the nitrogen atom within the ring.

One of the best known examples of a plant mutagen/carcinogen is cycasin, which occurs in the seeds of *Cycas circinalis.* Laqueur et al. (*49*) were the first to identify it and in 1967 proposed that it is a glucoside which is split by the β-glucosidase of gut flora to give a carcinogenic aglycone (*50*). Cycasin induces primarily hepatocarcinoma and occasional tumors of the kidney and intestine (*51–53*).

The mutagenicity of the aglycone (methylazoxymethanol) has been shown in *Salmonella typhimurium* (*54*) and in *Drosophila* (*44*). Another naturally occurring carcinogen is safrole, found in members of the Lauraceae and Aristolochiaceae. It is carcinogenic in rats (*55, 56*) and mice (*45, 56*). A metabolite of safrole, 1′-acetoxysafrole, has been shown to be mutagenic in the Ames test (*57*). Caffeine has been thoroughly studied and is found to induce mutation in *E. coli* (*58*) and *Drosophila* (*59*) and chromosomal aberration in *Allium cepa, Vicia faba,* and Chinese hamster. The most characteristic effect, however, seems to be an enhancement of cell killing and mutagenesis after treatment with other chemical or physical mutagens. Studies in both bacteria and eukaryotes indicate that this is the result of inhibition of DNA repair enzymes (*58*).

Bracken fern, *Pteridium aquilinum,* is associated in cattle with a haemorrhagic syndrome resembling the effects of radiation exposure (*60*). An investigation of the carcinogenic properties of bracken has established that it causes malignancies in laboratory rats and mice as well as sheep and cows (*61*). Recently, Wang et al. (*62*) have fractionated *Pteridium* extracts and found that the tannin fraction produced a high incidence of bladder carcinoma in mice. Other plant tannins have been implicated, because there seems to be a correlation between oral and esophageal cancer rates and the frequent use of herbal and medicinal teas (*45*). This has been investigated with medicinal plants from Curaçao and approximately half were oncogenic (*63, 64*).

In another experiment, tannin extracts of several plants native to South Carolina were tested in rats. Extracts from *Liquidambar styraciflua, Quercus falcata, Limonium nashii* and *Myrica cerifera* were found to cause tumors (*65*).

Another group of potent plant carcinogens are the

pyrollizidine alkaloids. These are found in the Gramineae in *Festuca* and *Lolium* and in the Leguminosae in *Crotalaria* and *Cytisus* but are most characteristic of *Senecio* in the Compositae (*42*). Members of this group of alkaloids are mutagenic in *Drosophila* (*66*) and *Aspergillus* (*43*) and induce chromosome breaks in *Allium cepa* (*56*), *Drosophila* and mammalian cell cultures (*43*). Pyrollizidine alkaloids extracted from *Senecio jacobaea* caused hepatocarcinoma in rats (*66*). Among individual members of this group retrorsine, isatidine, lasiocarpine and monocrotaline are carcinogenic (*45, 66*) and monocrotaline, lasiocarpine, and heliotrine are mutagenic (*44*). Pyrollizidine alkaloids require mixed function oxidase activation and it is thought that they may then act as bifunctional alkylating agents (*67*).

Furocoumarins or psoralens are another group of naturally occurring mutagen/carcinogens and have been reviewed by Scott, Pathak and Mohn (*68*). They are found mainly in the Umbelliferae and Rutaceae. They are photoreactive molecules and with exposure to light cause photodermatitis. Exposure to the near ultraviolet (365 nm) seems to be required for genetic activity also; 8-methoxypsoralen combined with radiation exposure increases tumor incidence in mice and is mutagenic in *Drosophila, T4 phage, E. coli,* and *Aspergillus*. It appears that damage may be due to crosslinking of the DNA by psoralen.

It is unknown at this point exactly which plants may be toxic or mutagenic in the various microbial assays in common use. However, in view of the broad range of secondary metabolites produced by plants, it can be appreciated that these are not trivial problems and are factors which must be examined early in the process of selecting a bioconcentrator.

A final subject to be considered is the action of bioconcentrator metabolism on the substances being accumulated. As mentioned above, the effects could be of two kinds, either activation of promutagens or degradation. The degradation of organic pesticides by plants is comparatively well studied. This will aid the study of mutagenicity due to pesticides. However, degradation of the broad spectrum of mutagen/carcinogens by plants has not, to the authors' knowledge, been explored. Likewise, knowledge of mutagen activation in plants is scanty, although Gentile and Plewa (*69*) have shown that atrazine is activated to a mutagenic intermediate in corn. Increased chromosome breaks result from exposure of *Vicia faba* to a nitrosamine (*70*). Since nitrosamimes require activation, this indicates that the necessary intermediates are being formed. However, caution must be exercised since the mechanism of chromosome breakage probably is different from that of mutation. Furthermore, in some plants activation may not be occurring since no effect is seen even with potent mutagens (*71*).

Conclusion

The detection of dispersed mutagens in ecosystems may be possible with bioassays based upon selected components of the indigenous biota as bioconcentrators coupled with routine microbial procedures for detecting mutagens. With the concern for the possible presence of dispersed mutagens in ecosystems, such bioassay procedures will be increasingly used. Prior to drawing conclusions from such bioassays, the researcher should be aware of certain inherent aspects of these procedures which may generate false positives (presence of either the necessary metabolite in the extract where the microbial test is a back mutation system from auxotrophy to prototrophy or the extraction of an endogenous mutagen) or false negatives (presence of microbial toxins in the extract, the inactivation of the mutagen by the bioconcentrator, or the selection of a mutagen assay with the wrong genetic end point). In spite of these obstacles, assays based upon bioconcentrators will have a significant role in screening ecosystems.

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